약학석사학위논문

The Role of Phosphogluconate Dehydrogenase in Nrf2-mediated Proliferation and Migration of Human Hepatoma HepG2 Cells

Nrf2 를 통한 HepG2 인간 간종양 세포의 증식과 전이에서 포스포글루콘산 탈수소효소의 역할 규명

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Abstract

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Nuclear factor-erythroid-2-related factor 2 (Nrf2) is a transcription factor that binds to the antioxidant response element (ARE) present in the promoter regions of its target genes, encoding mostly antioxidant enzymes and other cytoprotective proteins involved in stress response. Recent studies report that in contrast to its tumor suppressive functions in normal cells, Nrf2 facilitates tumor progression in some cancer cells. However, the molecular mechanism underlying the oncogenic properties of Nrf2 is not yet well understood. Previous studies have shown that Nrf2 also regulates the expression of some of the pentose phosphate pathway (PPP)-related enzymes. Among these, phosphogluconate dehydrogenase (PGD) is of particular interest since its key byproducts, ribulose-5-phosphate (Ru-5-P) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) are utilized as a precursor for nucleotide synthesis and as a reducing agent for cellular antioxidant defense, respectively. The present study was intended to explore

the potential role of PGD, as a target protein of Nrf2, in the proliferation and migration of human hepatoma, HepG2 cells. Notably, Nrf2 regulates the transcription of *PGD* through direct binding to the ARE in its promoter region. Knockdown of *Nrf2* or *PGD* significantly inhibited HepG2 cell proliferation and migration. Conversely, Nrf2 overexpression in HepG2 cells led to increased cell proliferation and migration, which was suppressed by silencing of *PGD*. While Nrf2 regulates PGD expression, knockdown of the gene encoding this enzyme downregulated the expression of Nrf2 and its target antioxidant enzymes, including heme oxgenase-1 and glutamate-cysteine ligase catalytic subunit at both transcriptional and translational levels. In particular, *PGD* knockdown upregulated Kelch-like ECH-associated protein 1 (Keap1) protein expression. Treatment of HepG2 cells with Ru-5-P gave rise to a decrease in Keap1 protein expression without influencing its mRNA transcript level. This was accompanied by upregulation of both *Nrf2* mRNA and HO-1 protein. Collectively, the current study shows that Nrf2 promotes hepatoma cell growth and migration through activation of *PGD* transcription and that the PGD product, Ru-5-P, induces Keap1 degradation to activate Nrf2 signaling. Thus, there seems to exist a positive-feedback loop between Nrf2 and PGD which is exploited by hepatoma cells for their survival.

Keywords: Nuclear factor-erythroid-2-related factor 2 (Nrf2), Phosphogluconate dehydrogenase (PGD), Antioxidant response element (ARE), Pentose phosphate pathway, Ribulose-5-phosphate (Ru-5-P), Human hepatoma HepG2 cells

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Introduction

Nuclear factor-erythroid-2-related factor 2 (Nrf2) is a transcription factor widely known to be involved in the cellular defense against oxidative stress by controlling the expression of genes responsible for antioxidant and stress responses[1,2]. Due to this, Nrf2 has been considered as a protective protein which became one of the prime targets for chemoprevention[3]. However, recent studies show that cancer cells also harness Nrf2 to facilitate tumor growth and promote chemoresistance[4]. Numerous cancer types were found to have mutations in Nrf2, or its inhibitor Kelch-like ECH-associated protein 1 (Keap1), which resulted in a constitutively high expression of Nrf2 in these cells[5,6]. Furthermore, Nrf2 was found to stimulate proliferation and invasiveness of various cancer cells[7–9]. Yet, the molecular mechanisms by which Nrf2 promotes cancer plasticity have not yet been fully elucidated. Nrf2 activates its target genes by binding to the antioxidant response element (ARE) present 5'-upstream of their promoter region[10]. AREs, also known as electrophile response element (EpRE), has a core sequence of 5′-TGAG/CNNNGC-3′[11]. In an oligonucleotide microarray performed to determine Nrf2-regulated genes, a series of metabolic enzymes were identified as Nrf2 products[12].

Metabolic reprograming is one of the ten hallmarks of cancer identified in Hanahan and Weinberg's review paper published in 2011[13]. A growing body of evidence shows that metabolic reprogramming is necessary in order to support cancer cell growth and proliferation[14–16]. The Warburg effect describes the phenomenon in which cancer cells rely on aerobic glycolysis, rather than

the more efficient mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes[17]. In addition, this shift provides the cells with important macromolecular precursors needed to support cell proliferation and tumor growth[18]. Also, rather than just an indirect phenomenon, it is directed by oncogenes in order to support anabolic growth in cancer cells[19].

Phosphogluconate dehydrogenase (PGD) is the third enzyme in the pentose phosphate pathway (PPP) which converts 6-phosphogluconate to ribulose-5-phosphate (Ru-5-P) through oxidative decarboxylation. During this process, one molecule of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) is also produced. NADPH is essential for both cellular defence against oxidative stress and for reductive biosynthesis, such as lipogenesis[20]. Ru-5-P can be converted by ribose-5-phosphate isomerase to ribose-5-phosphate which provides the backbone for nucleotides[21]. Increased activity and expression of PGD have been reported in several cancers including lung[22], thyroid[23], and cervical intraepithelial neoplasia[24]. Suppression of PGD was found to attenuate cancer cell proliferation through reduction of lipogenesis and RNA biosynthesis[25]. Likewise, PGD knockdown suppresses tumor growth by inducing senescence in lung cancer cells[26]. Additionally, PGD was found to be necessary for c-Met phosphorylation for the promotion of tumor cell invasiveness in lung cancer cells[22]. However, the mechanisms underlying oncogenic functions of PGD as a downstream target gene of Nrf2 remain elusive.

In this study, the role of PGD in cancer cell proliferation and migration induced by Nrf2 were investigated in human hepatoma HepG2 cells. Nrf2

promoted PGD transcription by directly binding to the ARE in the promoter region of PGD. Furthermore, PGD was found to be a crucial downstream target gene in the Nrf2-mediated proliferation and migration. Notably, PGD comprises a positive-feedback loop with Nrf2. PGD, through its byproduct Ru-5-P, was found to induce Keap1 degradation resulting in an upregulation of Nrf2 and its target genes.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and Antibiotic-Antimycotic (100X) were products of GIBCO BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from GenDEPOT (Barker, TX, USA). Primary antibodies against Nrf2, PGD, lamin B, Keap1, and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibody for HO-1 was bought from Enzo Life Sciences (Farmingdale, NY, USA). The primary antibodies for actin and GCLC were obtained from AbClon Inc (Seoul, South Korea). Antibody against p62 was purchased from Abcam (Cambridge, MA, USA). The primary anti-rabbit IgG, HRP-linked antibody was bought from Cell Signalling Technology (Beverly, MA, USA). TRIzol®, SYBR®safe DNA gel stain, Lipofectamine® RNAiMAX, and Lipofectamine® 2000 were provided by Invitrogen (Carlsbad, CA, USA). Polyvinylidene difluoride (PVDF) membranes were supplied by Gelman Laboratory (Ann Arbor, MI, USA). Protease inhibitor cocktail tablets were provided by Boehringer Mannheimm (Mannheimm, Germany). The protein assay dye (Bradford) reagent was supplied by Bio-Rad Laboratories (Hercules, CA, USA). Actinomycin D and Ru-5-P were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The bicinchonic acid (BCA) protein assay reagent was obtained from Pierce Biotechnology (Rockford, IL, USA). All other chemicals used were in the purest form available commercially.

Cell Culture

HepG2 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM medium supplemented with 10% v/v FBS and 100 units/mL antibiotic-antimycotic at 37°C in an incubator with humidified atmosphere of 95% air/5% CO_2.

Immunocytochemical Analysis

HepG2 cells transfected with either siControl or siPGD were plated on 8-well chamber slides at a density of 2 x 10^4 per well. After fixation with 4% formaldehyde for 15 min at 37^{o}C, cells were rinsed with PBST (Phosphate-buffer saline containing 0.1% Tween-20) and treated with 0.1% Triton X-100 in PBS for 5 min. Then, the cells were washed and blocked with 0.05% Triton X-100 in PBS containing 5% bovine serum albumin (BSA) at room temperature for 1 h. The cells were then washed with PBST and then incubated with diluted (1:200) primary antibody overnight at 4^{o}C. After washing with PBST, samples were incubated with FITC-conjugated anti-rabbit secondary antibody in PBST (1:1000) containing 5% BSA at room temperature for 1 h. Samples were washed with PBST and stained with DAPI (4',6-diamidino-2-phenylindole) in PBS (1:3000) for 15 min. The samples were then washed with PBST and examined under a fluorescent microscope (Nikon, Japan).

Migration assay

Two-well Culture-Inserts (Ibidi®) were attached to 12-well plates. HepG2 cells transfected with the appropriate reagent were seeded at a density of 2.5 x 10^4 cells on each well of the Culture-Insert. After 24 h, the silicon inserts were removed and photographed under the microscope. The cells were again photographed after 48 h to assess the closure of the wound gap.

Transfection

siRNA transient transfection

HepG2 cells were reverse transfected with siRNA. The following siRNAs were used for transfection: human negative-siRNA (5'-CCUCGUGCCGUUCCAUCAGGUAGUU-3'), siNrf2 (5'-AAGAGUAUGAGCUGGAAAAACTT-3', Invitrogen), siPGD#1 (5'-CUCUUCGGUUCUGCUCUGU-3', Bioneer), and siPGD#2 (5'-CUCACACCUAUGAACUCUU(dTdT)-3', Bioneer). siRNA was diluted in 1mL Opti-MEM and vortexed to mix. Lipofectamine® RNAiMAX reagent was then added to the mixture and the mixture was incubated for at least 20 min prior to addition to the freshly seeded cells in media without antibiotics and FBS. At 24 h after transfection, the media was changed to full media (with 10% FBS and 1% antibiotic-antimycotic). This is then incubated for another 48 h, after which cells were harvested for either mRNA extraction or protein analysis.

Plasmid transfection

HepG2 cells were transfected with pcDNA 3.1 plasmid carrying cDNA for Nrf2 and a Myc tag (Plasmid #21555; Addgene; Cambridge, UK) using Lipofectamine®2000 reagent. The transfection was done according to the manufacturer's instructions. An 'empty' vector, pcDNA 3.1 plasmid, was considered as mock or control.

Chromatin Immunoprecipitation (ChIP) Assay

For ChIP assays, cells were washed with PBS and crosslinked with a 1% formaldehyde solution for 30 min at room temperature (25 °C). The crosslinking reaction was stopped by the addition of glycine to 125mM final concentration. Cell lysates were sonicated to generate DNA fragments with the average size of 300 to 600 base pairs. This was followed by immunoprecipitation with indicated antibodies, which were bound to Protein Agarose A/G (Santa Cruz, CA, USA) by an overnight incubation prior to use. Bound DNA fragments were eluted and purified using the Biomedic® Plasmid DNA Miniprep Kit (Seoul, Korea). The collected DNA was then amplified by PCR. The primer pairs used are indicated in *Table 1*.

Western blot analysis

Cells were first gently washed with cold PBS. Lysis buffer was then added to the plate and the cells were scraped and collected in a tube. The collected cells were incubated for 20 min at 4°C with continuous vortexing. This was then centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was collected as whole cell lysate. For obtaining the cytosolic and nuclear extracts, buffer A and buffer C were used respectively. Pellets were resuspended in hypotonic buffer A [10mM HEPES (pH 7.9), 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT and 0.2mM PMSF] for 15 min on ice. After that 0.1% Nonidet P-40 was added and the mixture was incubated for less than 5 min. It was then centrifuged at 12,000 rpm for 5 min at 4^oC. The supernatant containing the cytosolic fraction was recovered and the pellets were rinsed twice with hypotonic buffer A and resuspended again in the hypertonic buffer C [20mM HEPES (pH 7.9), 1.5mM $MgCl_2$, 420mM NaCl, 0.5mM DTT, 0.2mM PMSF, 0.2mM EDTA and 20% glycerol]. After incubation for 1 h on ice, the mixture was centrifuged at 12,000 rpm for 15 min at 4^oC. The supernatant was collected as nuclear extract. The protein concentration of whole cell lysates was determined using the BCA protein assay kit. Protein samples from whole cell lysates were mixed with sodium dodecyl sulfate (SDS) loading dye and boiled at 99°C for 10 minutes. Protein samples were electrophoresed on SDS-polyacrylamide gel and transferred to PVDF membranes. The blots were then blocked with 5% fat-free dry milk in PBST buffer for 1 hour at room temperature. The membranes were then incubated with primary antibodies diluted in PBST at 4°C overnight. After incubation, the membranes were washed, followed by incubation with 1:5000

dilution of the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit or mouse; Zymed Laboratories) in 2% fat-free dry milk in PBST for 1 h, followed again by washing with PBST. Protein bands were visualized with the ECL substrate detection reagent using the LAS-4000 image reader (Fuji film).

Polymerase Chain Reaction (PCR)

Total RNA was isolated from HepG2 cells using TRIzol® (Invitrogen) according to the manufacturer's protocol. One microgram of the extracted RNA for each sample was reverse transcribed to complementary DNA with MLV reverse transcriptase at 42°C for 50 min and 72°C for 15 min. One μL of cDNA was used for further amplification in sequential reactions using Solg™ 2X Taq PCR Smart mix 1 (SolGent; Seoul, Korea). The mRNA expressions of target genes were checked. The primer sequences and conditions used for each PCR reaction are listed in Table 1.

Colony Formation Assay

The cells were transfected with the indicated expression vectors or siRNAs in a 60 mm plate as described above. After 72 h of transfection, the cells were washed, counted and plated at a density of 500 cells per well in a 6-well plate. Cells were further cultured for 10 days in standard conditions. The plates were washed with PBS, fixed with chilled methanol for 10 min, and stained with 0.1% crystal violet solution. Plates were imaged by LAS-4000 image reader (Fuji film) and colonies having more than 50 cells were counted.

Table 1. List of primer sequences used

	Primer Sequence (5'-3')		Annealing Temperature (^{o}C)	No. of Cycles
Target Gene	Forward	Reverse		
PCR				
NRF2	TTC AAA GCG TCC GAA CTC CA	AAT GTC TGC GCC AAA AGC TG	52	25
Hmox1	CTC CCA GGG CCA TGA ACT TT	GAC AGC TGC CAC ATT AGG GT	57	25
PGD	GCT CTT CGG TTC TGC TCT GT	CCA GTT CCC ACT TTT GCA GC	56	25
Keap1	CAG AGG TGG TGG TGT TGC TTA	AGC TCG TTC ATG ATG CCA AAG	57	25
GCLC	TGA AGG GAC ACC AGG ACA GCC	GCA GRG RGA ACC CAG GAC AGC	62	25
GAPDH	ACC ACA GTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA	57	24
ChIP Assay				
PGD-ARE	CCC CCT CTA ACA GGA AGG GT	ACC ACT TTT TCC CCC ATA GAC AA	57	35

Cell Viability Assay/MTT Assay

Transfected cells incubated for 72 hours were trypsinized and seeded at a density of 3.5 x 10^3 cells per well in a 96-well plate. After 48 h of incubation, the media was changed with a 0.5mg/mL of thiazolyl blue tetrazolinium bromide (Sigma, St. Loius, MO, USA) in DMEM (MTT). The cells were incubated with MTT for 3 h. MTT was then removed, and DMSO was added to solubilize the formazan crystals formed. The absorbance per well was measured at 570 nm using a micro-plate reader (Bio-Rad Laboratories; Hercules, California, USA).

Statistical Analysis

Results were expressed as the means ± SEM of at least three independent experiments. The statistical significance of the difference between two groups was evaluated using Student's *t* test. Analysis was performed using IBM SPSS Statistics (Version 23). Statistical significance was accepted at $p < 0.05$, unless otherwise indicated.

Results

Nrf2 directly regulates PGD transcription in HepG2 cells

Nrf2 has been shown to regulate the expression of metabolic enzymes in various cancer cell lines[7,12]. Knockdown of *Nrf2* with siRNA resulted in the inhibition of mRNA and protein expression of PGD **(Fig. 1A and 1B)**. The efficiency of *Nrf2* knockdown was monitored by measuring HO-1 protein levels, a well-known target of Nrf2[27]. Upon activation, the heterodimer Nrf2 bound to a small Maf protein interacts with the ARE in the regulatory region of its target genes[28,29]. Nrf2 has been found to directly bind to the ARE-region in *PGD* in A549 cells[7]. To confirm Nrf2 interaction with the ARE consensus sequence in the PGD regulatory region in HepG2 cells, a ChIP assay was performed **(Fig. 1C)**. The results show that Nrf2 induces expression of PGD by directly binding to the ARE found in its promoter region.

To explore whether Nrf2 is associated with the stability of *PGD* mRNA, HepG2 cells were treated with Actinomycin D (10 μg/mL) to inhibit mRNA synthesis. However, the degradation of *PGD* mRNA did not significantly differ between siControl and siNrf2 transfected cells, suggesting that Nrf2 is unlikely to be involved in the stabilization of the *PGD* mRNA **(Fig. 1D)**.

Nrf2 promotes cell proliferation and invasion of HepG2 cells

Fundamental marks of malignant cancer are the presence of proliferative and invasive phenotypes[30]. Nrf2 was silenced through transfection of an siRNA targeting *Nrf2*, while overexpression was achieved by transfection of the cell

with pcDNA 3.1-Nrf2-Myc plasmid. Previous studies showed that Nrf2 promotes proliferation and survival in several cell types including cancer cells[7,31,32]. To validate this in HepG2 cells, several tests including the MTT Assay and the clonogenic assay were performed. The silencing of Nrf2 by siNrf2 transfection attenuated cell proliferation and survival (**Fig. 2A** and **2B**) while Nrf2 overexpression provoked an opposite effect. Furthermore, HepG2 cells transfected with siNrf2 showed reduced migration at 48 h **(Fig. 2C)**. Conversely, opposite effects were observed in the group transfected with pcDNA 3.1-Nrf2-Myc plasmid compared to the group transfected with an empty vector **(Fig 2D, E** and **F)**.

PGD is important for proliferation and migration in HepG2 cells

Rapid proliferation of cancer cells requires not only ATP but also building blocks necessary for the synthesis of lipids, nucleotides and amino acids[14]. The byproducts of PGD are NADPH, used for biosynthesis of many important cellular components, and Ru-5-P, a precursor in nucleic acid biosynthesis[25]. HepG2 cells were transfected with siControl or siPGD and cell proliferation and survival were evaluated using the MTT assay **(Fig 3A)** and the clonogenic assay **(Fig. 3B),** while migration was assessed by the use of wound-healing migration assay (**Fig. 3C**). *PGD* knockdown resulted in a decreased proliferation and migration of HepG2 cells. In addition, PGD was overexpressed in HepG2 cells by transfection of a PGD expression plasmid and proliferation was measured using the MTT assay **(Fig. 3D)** and the clonogenic assay **(Fig. 3E)**. Overexpression of PGD resulted to an increased proliferation

Nrf2 regulates cell proliferation and migration through PGD upregulation

Cells transfected with either pcDNA 3.1 (Mock) or pcDNA 3.1-Nrf2-Myc plasmid (Nrf2 plasmid) were co-transfected with either siControl or siPGD **(Fig. 4)**. Nrf2 plasmid-siControl co-transfected cells had an increased proliferation compared to the Mock-siControl co-transfected cells. However, this was inhibited by the knockdown of PGD in the Nrf2 plasmid transfected cells (Nrf2 plasmid-siPGD) **(Fig. 4A)**. Given that the increase in cell proliferation due to Nrf2 over-expression was hindered by silencing of PGD, this finding suggests that PGD is required by Nrf2 in the promotion of cell proliferation of HepG2 cells. Similar results were observed when these groups were subjected to a wound healing assay. The increased migratory capability observed in Nrf2 plasmid-siControl was attenuated in Nrf2 plasmid-siPGD cells which suggests that Nrf2-mediated migration also requires PGD **(Fig. 4B)**.

PGD regulates Nrf2 through a positive-feedback loop

A previous study provided evidence that glucose-6-phosphate dehydrogenase (G6PD), the first enzyme in the PPP, and NADPH are important in the expression of Nrf2-initiated antioxidant proteins, HO-1 and glutathione reductase[33]. Since PGD is part of the PPP, I first examined its effect on the transcription and translation of Nrf2 target genes. Well recognized target genes of Nrf2 are antioxidant and phase II enzymes which include HO-1[34] and the catalytic subunit of GCLC[35]. Knockdown of PGD downregulated their mRNA (*Hmox1* and *GCLC*) and protein (HO-1 and GCLC) expressions **(Fig. 5A and**

5B). Surprisingly, the mRNA level of *Nrf2* was also downregulated in HepG2 cells transfected with siPGD **(Fig. 5C)**. In addition, the expression of Nrf2 in Nrf2-overexpressing HepG2 cells was attenuated after siPGD co-transfection compared to the control **(Fig. 5D)**. Furthermore, subcellular fractionation **(Fig. 5E)** and immunocytochemistry **(Fig. 5F)** showed that knockdown of PGD decreased the nuclear localization of Nrf2.

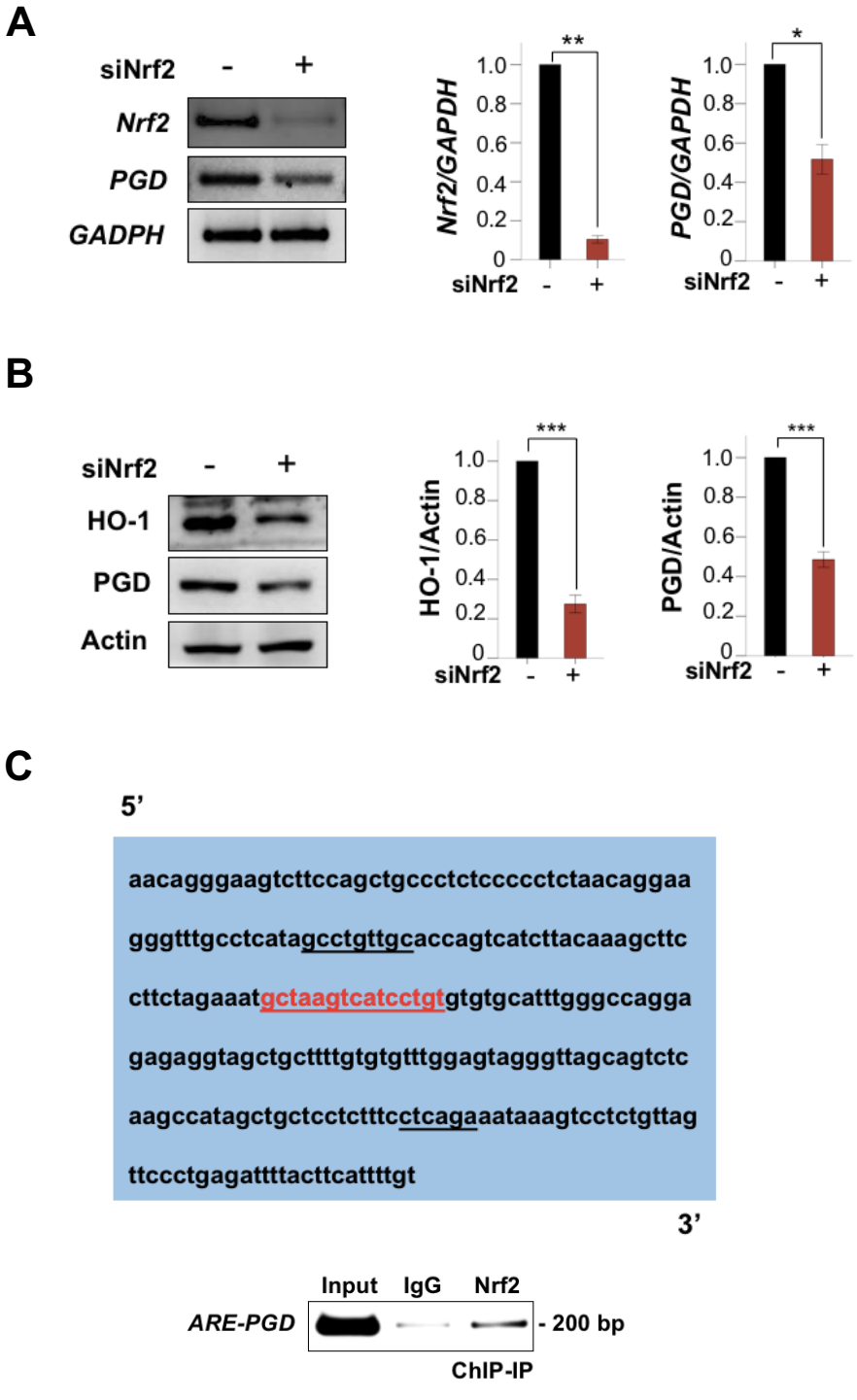
PGD promotes the degradation of Keap1, a negative regulator of Nrf2

Keap1 is a negative regulator of Nrf2[36]. It interacts with the N-terminal Neh2 domain of Nrf2 to facilitate its proteolytic degradation[37]. *PGD* silencing upregulated Keap1 protein (**Fig. 6A**), but not mRNA (**Fig. 6B**) levels. This suggests that PGD promotes the degradation of Keap1 protein primarily via post-transcriptional mechanisms. Previous studies have shown degradation of Keap1 through p62-dependent autophagy[38]. Of note, knockdown of *PGD* decreased p62 protein levels **(Fig. 6C)**. This suggests that p62-dependent degradation of Keap1 may be one mechanism underlying the positive-feedback loop between PGD and Nrf2.

Ru-5-P increases mRNA expression of *Nrf2* and upregulates HO-1 protein levels

PGD converts 6-phosphogluconate to Ru-5-P which then enters the non-oxidative arm of the PPP and proceeds to purine biosynthesis[39]. To determine whether this metabolite affects the Nrf2 regulatory network, HepG2 cells were treated with increasing doses (400, 600, and 800 μM) of Ru-5-P for 24 h. Ru-

5-P treatment increased Nrf2 mRNA levels in a dose-dependent manner **(Fig. 7A)**. As an indicator of Nrf2 activity, HO-1 protein levels were measured by Western blot analysis. As seen in **Fig. 7B**, there was a dose-dependent upregulation of HO-1 following Ru-5-P treatment. Moreover, the concurrent downregulation of Keap1 protein **(Fig. 7C)** supports the notion that the positive feedback mechanism between PGD and Nrf2 is mediated, at least in part, by Ru-5-P.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PGD
GADPH
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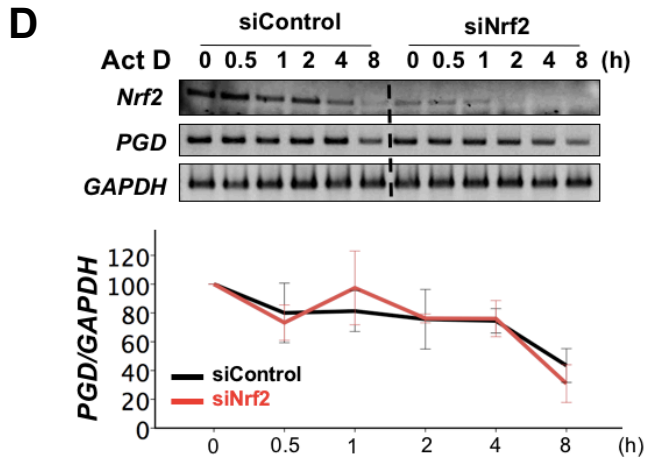
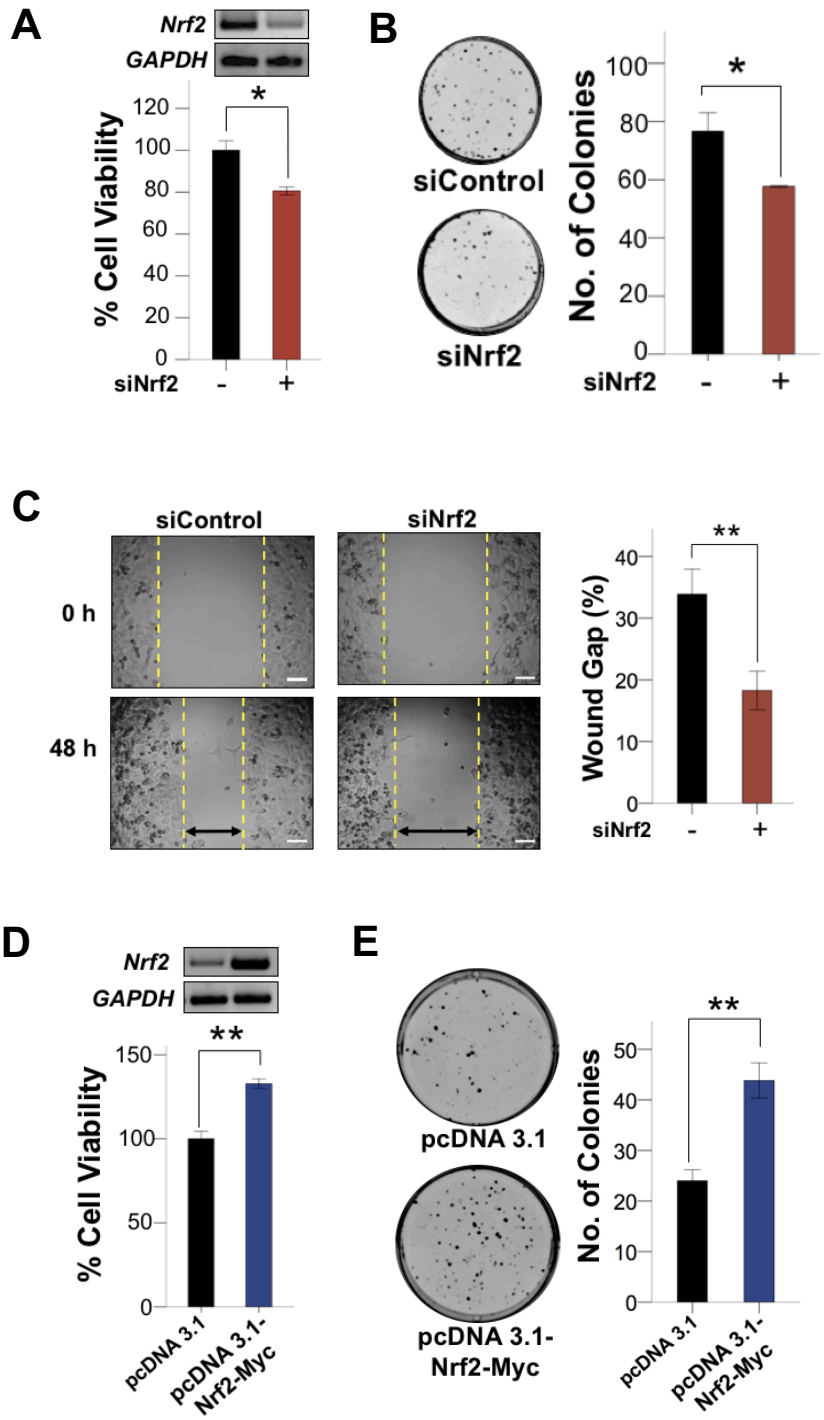


Figure 1. Nrf2 regulates the transcription of *PGD* by binding to the ARE in its promoter region. HepG2 cells were transfected with either a non-targeting siRNA or siNrf2 and incubated for 72 h. **(A)** The expression of *PGD* was determined by PCR. Relative mRNA levels were normalized to *GAPDH*. **(B)** Total cell lysates were evaluated by Western blot analysis. Band intensities were normalized to actin. **(C)** ChIP assay was performed on Widl-type HepG2 cells to detect the binding of Nrf2 to the ARE in the promoter region of *PGD*. Protein-DNA complexes were stabilized by crosslinking. Nrf2 was immunoprecipitated from the lysate. Reverse crosslinking was performed and DNA was extracted and purified. Samples were amplified through PCR. Band intensities were normalized to *GAPDH*. **(D)** Transfected cells were treated with actinomycin D (10 μg/mL) for 0, 0.5, 1, 2, 4, and 8 h. Mean band intensities normalized to *GAPDH* was divided by the 0 h control. The mean from each time point was plotted vs. time. For all panels, each bar shows the mean of three independent experiments ± SEM. The *p*-values were determined by independent *t*-test (*, $0.05 > p > 0.01$; ** , $0.01 > p > 0.001$; *** , $0.001 > p$ vs. control).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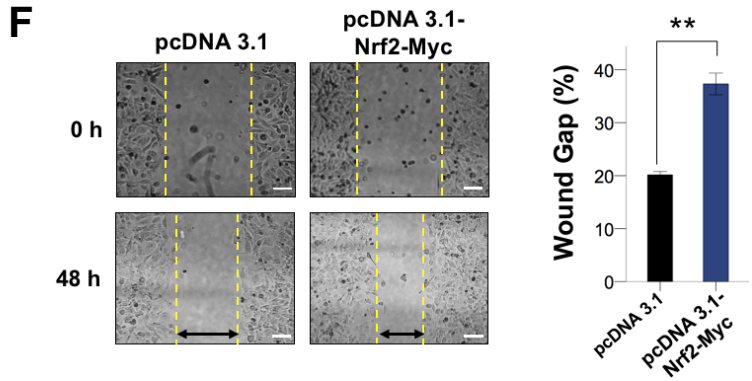
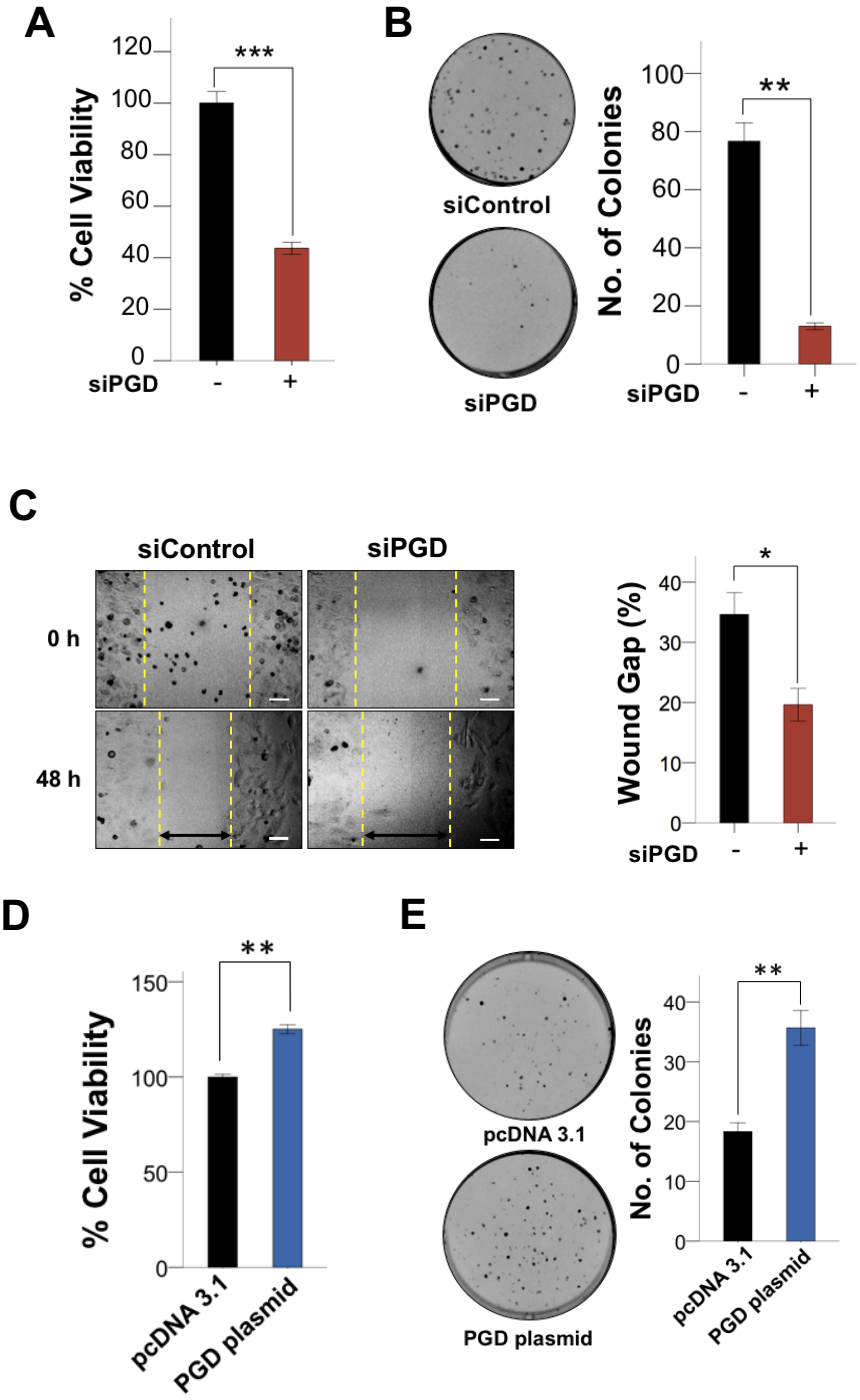


Figure 2. Nrf2 mediates proliferation and migration in HepG2 cells. (A to **C)** HepG2 cells were transfected with either a non-targeting siRNA or siNrf2 **(A)** After 48 h of incubation, cells were seeded in a 96-well plate. Cell viability was measured by the MTT assay 48 h after seeding. **(B)** After 48 h of incubation, cells were seeded in a 6-well plate. Formed colonies were counted after 10 days of incubation. **(C)** Cells were seeded into the two wells of an insert. After 24 h, the insert was removed and the cells were incubated for 48 h more. The wound gap was measured under a light microscope. **(D** to **F)** HepG2 cells transfected with pcDNA 3.1 (Control) or pcDNA 3.1-Nrf2-Myc (Nrf2-expressing plasmid). **(D)** After 24 h of incubation, cells were seeded in a 96-well plate. Cell viability was measured by the MTT assay 48 h after seeding. **(E)** After 24 h of incubation, cells were seeded in a 6-well plate. Formed colonies were counted after incubation for 10 days. **(F)** Cells were seeded into the two wells of an insert. After 24 h, the insert was removed and the cells were incubated for 48 h more. The gap was measured under a light microscope. For all panels, each bar shows the mean of three independent experiments ± SEM. The *p*-values were determined by independent *t*-test (*, $0.05 > p > 0.01$; ** , $0.01 > p > 0.001$; *** , $0.001 > p$ vs. control. Scale bar represents 100 µm.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pcDNA 3.1
PGD plasmid

Figure 3. PGD is involved in HepG2 cell proliferation and migration. (A to C) HepG2 cells were transfected with either a non-targeting siRNA or siPGD. **(A)** After 48 h of incubation, cells were seeded in a 96-well plate. Cell viability was measured by the MTT assay 48 h after seeding. **(B)** After 48 h of incubation, cells were seeded in a 6-well plate. Colonies were counted after 10 days of incubation. **(C)** Cells were seeded into the two wells of an insert. After 24 h, the insert was removed and the cells were incubated for 48 h more. The wound gap was measured under a microscope. **(D** to **E)** HepG2 cells transfected with pcDNA 3.1 (Control) or PGD plasmid (PGD-expressing plasmid). **(D)** After 24 h of incubation, cells were seeded in a 96-well plate. Cell viability was measured by the MTT assay 24 h after seeding. **(E)** After 24 h of incubation, cells were seeded in a 6-well plate. Formed colonies were counted after incubation for 10 days. For all panels, each bar shows the mean of three independent experiments ± SEM. The *p*-values were determined by independent *t*-test (*, $0.05 > p > 0.01$; ** , $0.01 > p > 0.001$; *** , $0.001 > p$ vs. control). Scale bar represents 100 μm.

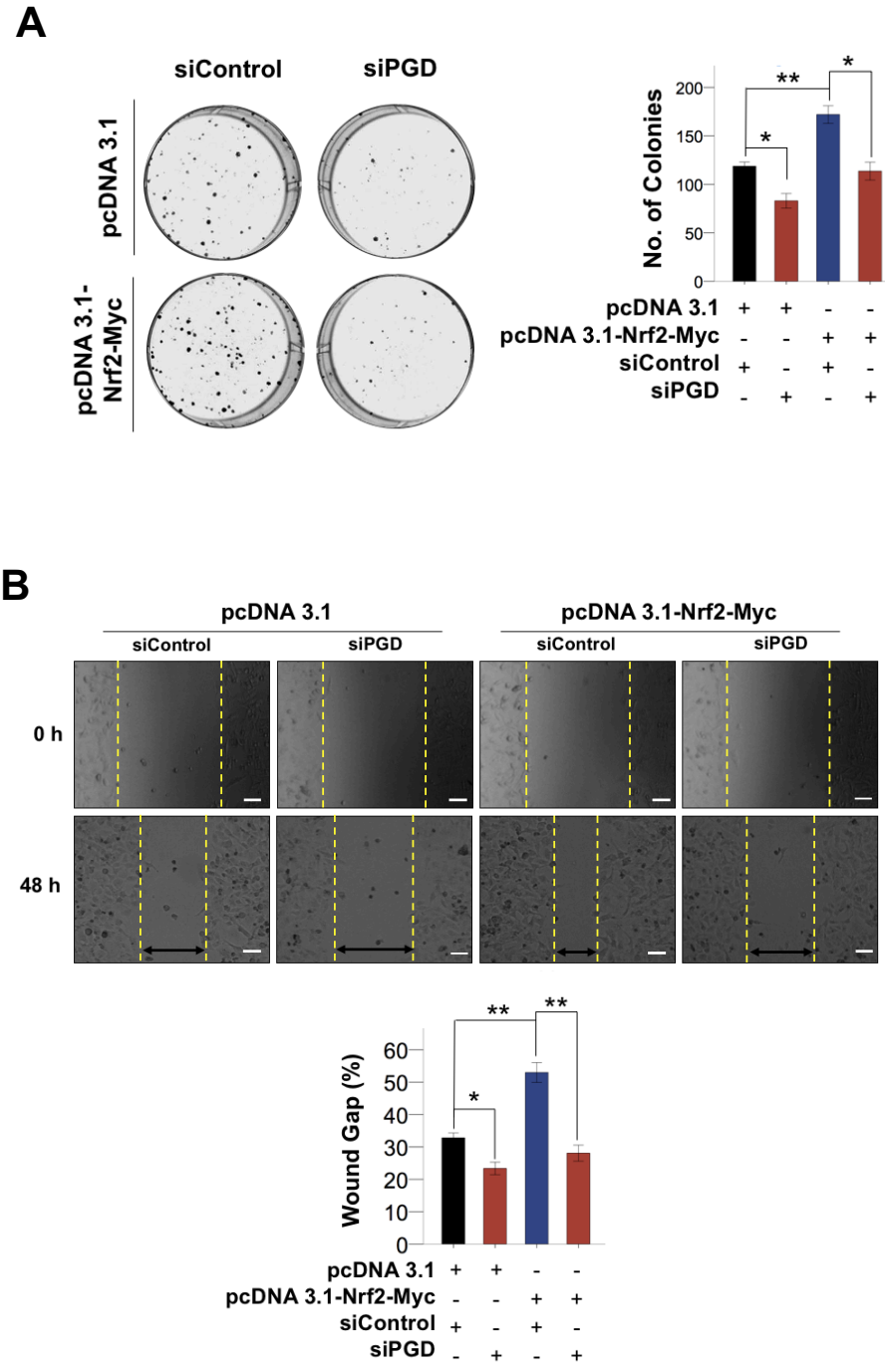
A
siControl
siPGD
pcDNA 3.1
pcDNA 3.1-Nrf2-Myc
No. of Colonies
200
150
100
50
0
**
*
*
pcDNA 3.1 + + - -
pcDNA 3.1-Nrf2-Myc - - + +
siControl + - + -
siPGD - + - +
B
pcDNA 3.1
pcDNA 3.1-Nrf2-Myc
siControl
siPGD
siControl
siPGD
0 h
48 h
Wound Gap (%)
60
50
40
30
20
10
0
**
**
*
pcDNA 3.1 + + - -
pcDNA 3.1-Nrf2-Myc - - + +
siControl + - + -
siPGD - + - +

Figure 4. PGD is involved in Nrf2-mediated HepG2 cell proliferation and migration. HepG2 cells were transfected with either a non-targeting siRNA or siPGD and incubated for 24 h. Each group was then subsequently co-transfected with either pcDNA 3.1 (Control) or pcDNA 3.1-Nrf2-Myc (Nrf2-expressing plasmid). **(A)** After 48 h of incubation, cells were seeded in a 6-well plate. Formed colonies were counted after 10 days of incubation. **(B)** Cells were seeded into the two wells of an insert. After 24 h, the insert was removed and the cells were incubated for 48 h more. The wound gap was measured under a microscope. For all panels, each bar shows the mean of three independent experiments ± SEM. The *p*-values were determined by independent *t*-test (*, $0.05 > p > 0.01$; ** , $0.01 > p > 0.001$; *** , $0.001 > p$ vs. control). Scale bar represents 100 μm.

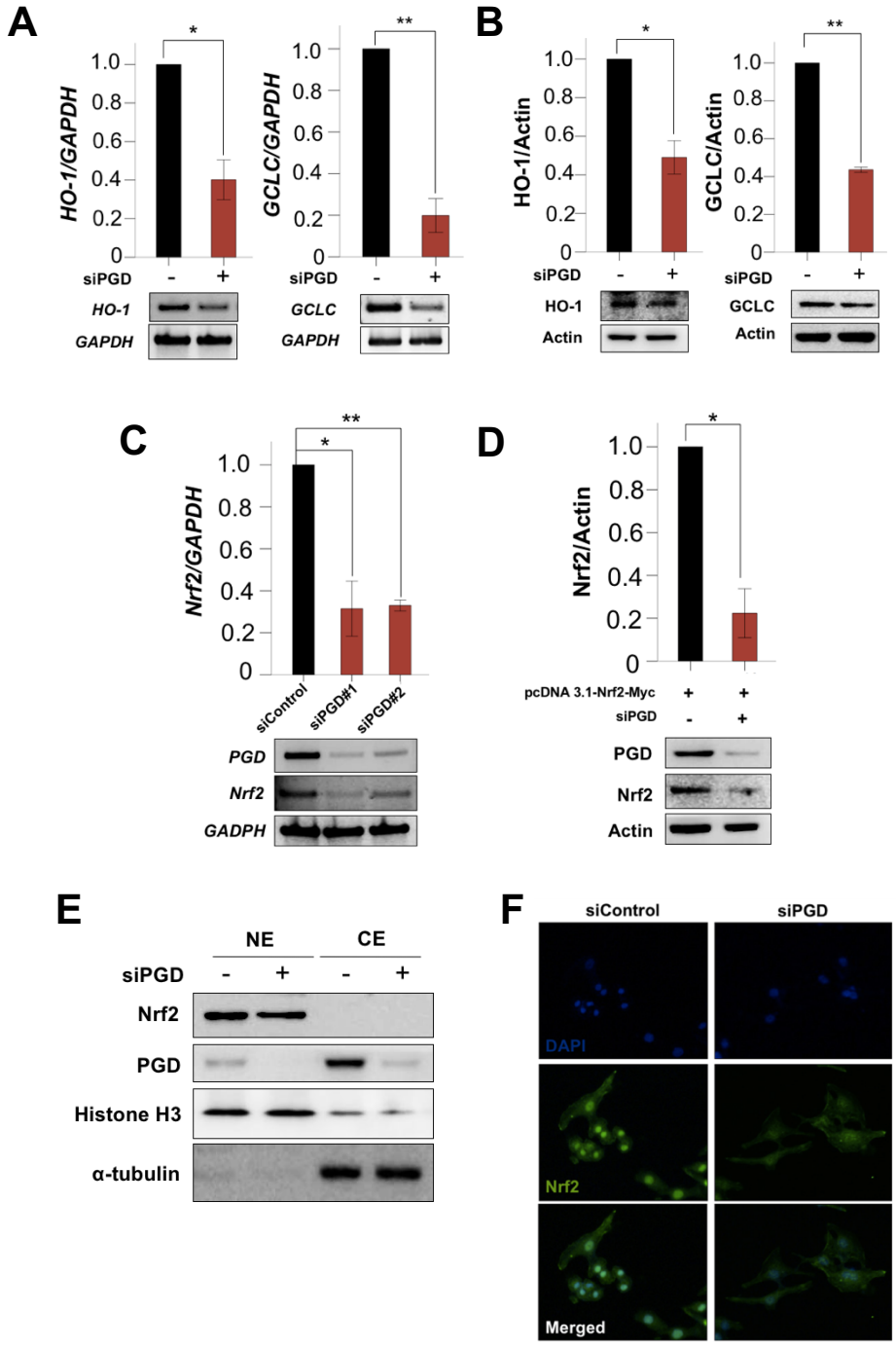
A
HO-1/GAPDH
GCLC/GAPDH
siPGD
HO-1
GCLC
GAPDH
B
HO-1/Actin
GCLC/Actin
Actin
C
Nrf2/GAPDH
siControl
siPGD#1
siPGD#2
PGD
Nrf2
GADPH
D
Nrf2/Actin
pcDNA 3.1-Nrf2-Myc
E
NE
CE
Histone H3
α-tubulin
F
siControl
siPGD
DAPI
Nrf2
Merged

Figure 5. PGD regulates Nrf2 expression through a positive-feedback loop. HepG2 cells were transfected with either a non-targeting siRNA or siPGD siRNA sequences and incubated for 72 h. **(A)** mRNA levels of Nrf2 target genes were evaluated by RT-PCR. Band intensities were normalized to *GAPDH*. **(B)** Protein levels of Nrf2 target genes were evaluated by RT-PCR. Band intensities were normalized to actin. **(C)** The mRNA levels of Nrf2 were determined by RT-PCR. Obtained bands were normalized to *GAPDH*. **(E)** Cells transfected with pcDNA 3.1-Nrf2-Myc were co-transfected with either a non-targeting siRNA or siPGD. Total lysates were evaluated by Western blot analysis. Band intensities were normalized to actin. **(E)** siControl or siPGD transfected cells were subjected to subcellular fractionation and analyzed by Western blot analysis. **(F)** Cells were fixed and the localization of Nrf2 was visualized by immunofluorescence staining. For all panels, each bar shows the mean of three independent experiments ± SEM. The *p*-values were determined by independent *t*-test (*, $0.05 > p > 0.01$; ** , $0.01 > p > 0.001$; *** , $0.001 > p$ vs. control).

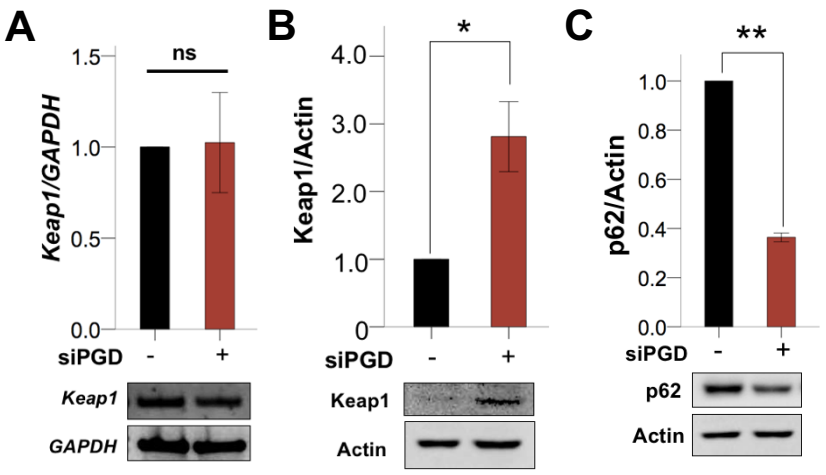

Figure 6. PGD promotes the protein degradation of Keap1. **(A** to **C)** HepG2 cells were transfected with either a non-targeting siRNA or siPGD. **(A)** The expression of *Keap1* was determined by PCR. Relative mRNA levels were normalized to *GAPDH*. (B to C) Total cell lysates were evaluated by Western blot analysis. **(B)** The band intensities of Keap1 were normalized to actin. **(C)** The protein level of p62 was determined by Western blot analysis and values were normalized to actin. For all panels, each bar shows the mean of three independent experiments ± SEM. The *p*-values were determined by independent *t*-test (*, $0.05 > p > 0.01$; ** , $0.01 > p > 0.001$; *** , $0.001 > p$ vs. control). ns, not significant.

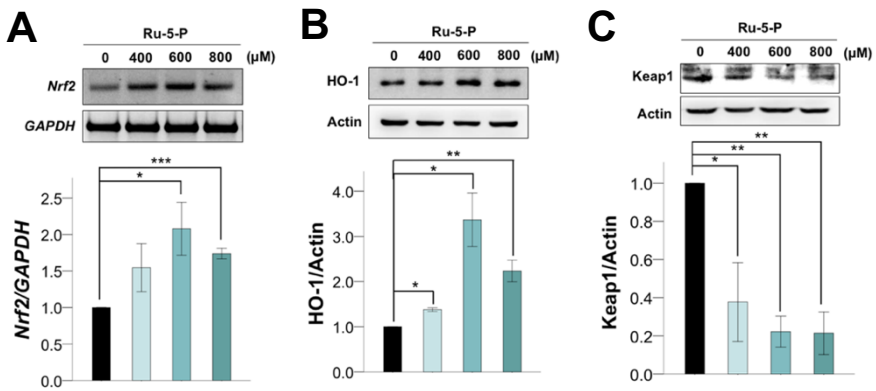

Figure 7. Ru-5-P regulates the positive feedback loop between PGD and Nrf2. HepG2 cells were treated with 0, 400, 600, and 800 μM of Ru-5-P for 24 h. **(A)** The mRNA level of *Nrf2* was determined by PCR. Relative mRNA levels were normalized to *GAPDH*. **(B** to **C)** Total cell lysates were evaluated by Western blot analysis. **(B)** The band intensities of HO-1 and **(C)** Keap1 were normalized to actin. For all panels, each bar shows the mean of three independent experiments ± SEM. The *p*-values were determined by independent *t*-test (*, $0.05 > p > 0.01$; ** , $0.01 > p > 0.001$; *** , $0.001 > p$ vs. control).

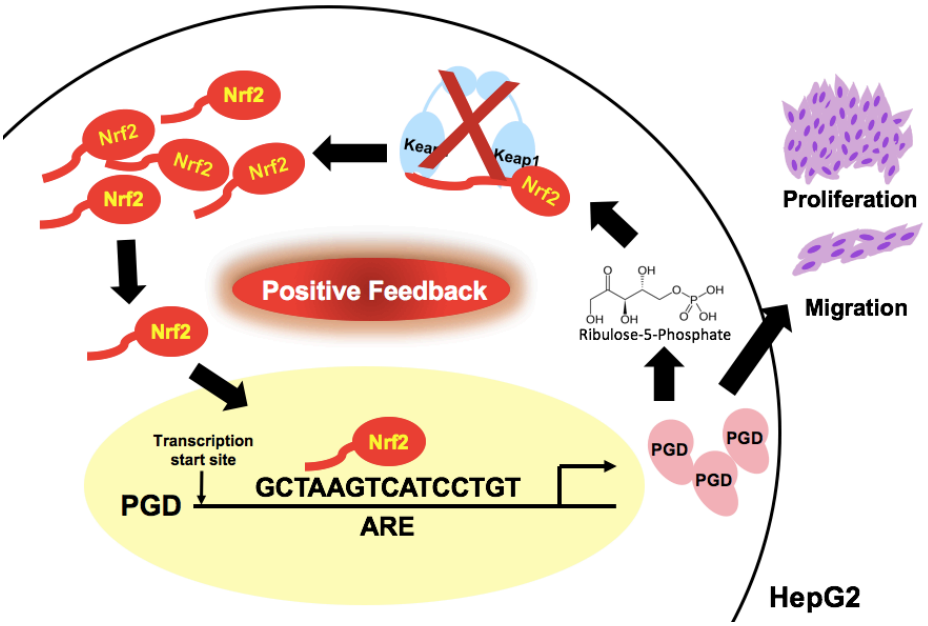

Figure 8. A schematic representation of the mechanism underlying the proposed positive-feedback loop between Nrf2 and PGD. Nrf2 binds to the ARE consensus sequence present in the PGD regulatory region, thereby promoting its transcription in HepG2 cells. The increased PGD expression leads to hepatoma cell proliferation and migration. Moreover, the increase in the PGD product Ru-5-P, induces the degradation of Keap1 to create a positive feedback loop between PGD and Nrf2.

Discussion

Two decades have passed since the function of Nrf2 as a regulator of antioxidant response and hence, a protector from environmental insults and disease development was discovered[40]. Damage to DNA, proteins, or lipids due to oxidative stress has been linked to inflammation and cancer, among other diseases[41]. *Nrf2*-knockout mice were found to be more susceptible to environmental carcinogens[42,43]. Furthermore, sensitivity to the chemoprotective effects of various phytochemicals was diminished in *nrf2*-knockout mice[44,45]. On the other hand, mutations to either Nrf2 or its regulatory proteins were found to lead to its aberrant overactivation in several cancers such as, epithelial, squamous cell[46], renal[47], lung[48], breast, prostate[49] and liver cancer[50]. Nrf2 provides cancer cells with ample reducing power to maintain ROS at levels to promote their growth and survival[51]. Evidence supporting the effect of Nrf2, either protective[52–54] or harmful[55,56], are still being published in the recent years. Thus, there is a need to conduct additional research in order to more accurately predict the function of Nrf2 in a defined disease state. In order to better understand the biological role of a transcription factor, it is important to identify and characterize its target genes[57]. I showed through ChIP assay that Nrf2 regulates *PGD* through binding to the ARE in the regulatory region of *PGD* in HepG2 cells. This finding is consistent with the result of a previous study done with lung cancer cells[7].

Increasing evidence shows that cancer is also a metabolic disease[58]. Metabolic reprogramming in cancer cells supports bioenergetics and the

macromolecular synthesis needed in their proliferation which accompany activation of distinct signal transduction pathways and transcriptional network reorganization[59]. The high catalytic activity of PGD has been associated with poor relapse-free survival times in breast cancer[60]. Elevated PGD expression levels also correlate with an advancing stage of lung carcinoma. Notably, PGD was found to be required in the phosphorylation of c-Met to promote migration of lung carcinoma cells[22]. Previous studies have demonstrated that the inhibition of cancer cell proliferation upon PGD knockdown is mediated through a senescence-associated mechanism[22,26]. Moreover, it has been suggested that inhibition of proliferation upon PGD knockdown may be due to accumulated glucose metabolites. In one study, it was shown that the suppression of cell proliferation due to PGD silencing was recovered upon silencing G6PD[26].

Nrf2 target genes have been shown to partake in cell growth and tumorigenesis. HO-1, one of the most well-known targets of Nrf2, was shown to be involved in the metastasis[61], invasion[62], and inhibition of apoptosis[63] in various cancer types. Similarly, NQO1 overexpression, which is found to be elevated in several solid tumors[64,65], also plays a role in the growth and chemoresistance of tumors[66–68]. In addition, higher protein levels of GCLC were found in colorectal tumor tissue compared to adjacent normal tissue[69], and this has been implicated in the development of tamoxifen-resistant breast cancer[70]. Unlike these Nrf2-regulated antioxidant enzymes, there are relatively few reports describing the signals related to PGD as a modulator the tumorigenic processes mediated by Nrf2. The present study provides convincing evidence

supporting the involvement of PGD in Nrf2-mediated proliferation and migration in HepG2 cells. Furthermore, this study proposes a positive-feedback loop between Nrf2 and PGD as the underlying mechanism regulating these processes.

In normal conditions, Keap1, a cysteine-rich cytoskeleton binding protein, binds to Nrf2 in the cytoplasm inhibiting its translocation to the nucleus[71]. It also facilitates the degradation of Nrf2 by serving as an adaptor between Nrf2 and the Cullin3-based E3-ligase ubiquitylation complex[72]. In hepatocytes, one mechanism of Keap1 degradation involves p62-dependent autophagy[38]. The DPSTGE domain in p62 interacts with arginine residues at the Kelch domain of Keap1, the same domain of Keap1 that interacts with Nrf2[73]. p62 then binds ubiquitin and LC3 and acts as a substrate for selective autophagy[74]. This present study has shown that silencing *PGD*, increases Keap1 and decreases p62 protein levels. These results suggest that the positive feedback loop between Nrf2 and PGD is facilitated by the destabilization of Keap1 protein. However, the involvement of p62-dependent autophagic degradation of Keap1 needs further confirmation.

Oncometabolites are endogenous metabolites whose abnormal accumulation contributes to the growth and metastasis of tumors[75]. Several oncometabolites have already been identified and many of these play a role in the control of cell division processes[76–78]. PGD activity produces Ru-5-P and NADPH as byproducts. NADPH is vital for the protection of cells from ROS-induced oxidative damage by providing a reducing power [20]. However, the magnitude of the effect of PGD in the regulation of overall NADPH production

in cancer cells is still in question. It has been reported that although PGD knockdown increases ROS levels in the cell, inhibition of PGD does not alter the steady-state level of NADPH[26]. On the other hand, Ru-5-P is a precursor for nucleotide synthesis. Interestingly, Ru-5-P was found to inhibit AMPK activation and hence, activate lipogenesis through disruption of the active LKB1 complex[79]. The results of this study indicate that Ru-5-P plays a role in the positive feedback loop between PGD and Nrf2. The results also demonstrate that Ru-5-P may be a potential oncometabolite in hepatocellular carcinoma.

This study demonstrates, for the first time, the existence of a positive-feedback loop between PGD and Nrf2. In leukemia cells, targeting PGD was proven to be selective and nontoxic to normal cells[25]. Hence, the positive feedback loop between PGD and Nrf2 opens an exciting possibility of attenuating Nrf2 signalling by targeting PGD in cancer cells, especially for those which exhibit chemoresistance due to Nrf2 overactivation. Considering that the role of Nrf2 in cytoprotection is still debatable, targeting PGD may be a good alternative strategy and should be examined in future studies.

In summary, Nrf2 regulates the expression of PGD by directly binding to the ARE in its regulatory region. Moreover, the regulation of PGD by Nrf2 plays an important role in Nrf2-mediated tumor plasticity through stimulation of cell proliferation and migration. Notably, the PGD product Ru-5-P, is suggested to induce these oncogenic effects by inducing activation of Nrf2, and subsequently upregulation of its target genes, in a positive-feedback loop. Taken together, these results suggest the possibility of PGD as a potential target in inhibiting tumor growth in hepatocellular carcinoma. However, in order to

utilize this pathway for cancer treatment, further mechanistic and clinical studies are needed.

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Nrf2 를 통한 HepG2 인간 간종양 세포의 증식과 전이에서 포스포글루콘산 탈수소효소의 역할 규명

Nuclear factor-erythroid-2-related factor 2 (Nrf2)는 항산화 효소 및 기타 스트레스 반응과 연관된 다른 세포 보호 단백질을 암호화하는 유전자의 프로모터 영역에 있는 antioxidant response element (ARE) 와 주로 결합하는 전사인자이다. 정상 세포에서 Nrf2 는 암화 과정을 억제하지만, 일부 암 세포에서는 암화 과정을 촉진하는 것으로 알려졌다. 하지만, 아직까지 Nrf2 의 종양 생성 능력을 가름하는 분자적 메커니즘은 정확히 알려지지 않았다. 선행 연구를 통해 우리는 Nrf2 가 5 탄당 인산화경로 (Pentose phosphate pathway) 관련 효소의 발현을 조절하는 것을 확인하였다. 포스포글루콘산 탈수소효소 (phosphogluconate dehydrogenase, PGD)는 뉴클레오티드 합성의 전구체인 리블로스 -5-인산 (Ru-5-P)과 세포항산화방어에 필요한 NADPH 를 핵심 부산물로 생성한다.

본 연구자 Nrf2 의 표적 유전자인 PGD 가 인체 간암 HepG2 세포의 증식과 전이에 미치는 역할을 규명하고자 하였다. Nrf2 는 PGD 의 ARE 영역에 직접 결합함으로써 PGD 의 전사를 조절하였다. Nrf2 혹은 PGD 의 유전자를 저해하였을 때 HepG2 세포의 증식과 침투성이 억제되었으나, Nrf2 의 과발현은 PGD 의 유전자를 silencing 함으로써 억제되었던 HepG2 세포의 증식과 전이능을 복원시켰다. PGD 유전자 억제는 Nrf2 와 그의 하위 유전자인 heme oxgenase-1, NAD(P)H quinine oxidoreductase 1 과 glutamate-cysteine ligase catalytic subunit 의 mRNA 및 단백질 발현을 동시에 감소시켰다. 또한, PGD 유전자를 억제했을 때 Nrf2 의 핵내 이동도

감소하였다. 결론적으로, 본 연구에서는 Nrf2 는 PGD 의 발현을 증가시켜 간종양 세포의 증식과 전이를 촉진시키며, PGD 는 자신의 유전자 발현을 조절하는 Nrf2 신호 전달 체계를 활성화함을 확인하였다. 이는 Nrf2 와 PGD 사이에 양성 되먹임 고리(positive feeback loop)가 존재함을 시사하며, 간암 세포는 생존을 위해 이 메커니즘을 이용하는 것으로 사료된다.

주요어 : Nuclear factor-erythroid-2-related factor 2 (Nrf2), 포스포글루콘산 탈수소효소 (Phosphogluconate dehydrogenase, PGD), 항산화 반응 효소 (Antioxidant response element), 5 탄당 인산화경로 (Pentose phosphate pathway, PPP), Ribulose-5-phosphate (Ru-5-P), 인간 간종양 HepG2 세포

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